EX. 2

NOTE

THE REACTION OF CYSTEINE AND RELATED COMPOUNDS WITH PENICILLINS AND CEPHALOSPORINS*

EUGENE S. WAGNER** and MARVIN GORMAN

Lilly Research Laboratories,
Eli Lilly and Company,
Indianapolis, Indiana, U.S.A.

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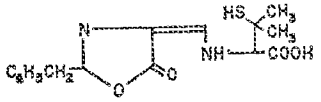
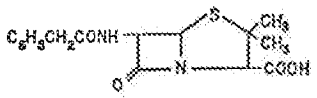
Evidence has been published for the reaction of penicillenic acid[1,2] and for the direct interaction of penicillin[3,4,5] with free amino groups of protein to form penicilloyl compounds. These penicilloyl derivatives are reported to be the major determinant in penicillin allergy[3,5].

Our initial observation that benzylpenicillenic acid, one of the major degradation products of benzylpenicillin, is converted by ethanethiol to α-ethylthiobenzylpenicilloate[7] generated an interest in the action of thiol-containing compounds on the β-lactam of penicillins. The possibility that benzylpenicillenic acid or benzylpenicillin, by reaction with the sulfhydryl groups of protein, could form a determinant responsible for an allergic response is of theoretical interest.

A comparison of the reactivity of benzylpenicillin and of benzylpenicillenic acid with thiol-containing nucleophiles was undertaken. The progress of the reactions was monitored spectrophotometrically. Within minutes the reaction of benzylpenicillenic acid with compounds containing free sulfhydryl groups is complete (Table 1). The results indicate that a large variety of compounds react, and only the absence of a free sulfhydryl interferes with reactivity. As reported earlier[6] the reaction of benzylpenicillin with 2-mercaptoamines having both a sulfhydryl group and an amino group also is complete within minutes. When either the sulfhydryl or the amino group of the 2-mercaptoamine is absent or blocked, no reaction is observed with benzylpenicillin.

Since the ability to form penicilloyl-thioesters differs with benzylpenicillenic acid and benzylpenicillin, we investigated the reactivity of several cephalosporin antibiotics with 2-mercaptoamines. The stabilities of a variety of penicillins and cephalosporins in the presence of cysteine were measured (Table 2). At pH 7.5 and room temperature, both 3-methyl cephem and 3-acetoxymethyl cephem derivatives are unreacted after four

Table 1. Reaction of benzylpenicillenic acid and benzylpenicillin with thiol-containing compounds

Compound present in reaction mixture	 Benzylpenicillenic acid	 Benzylpenicillin
$H_2NCH_2CH_2SH$	+	+
H_2NCHCH_2SH COOH	+	+
$HOCH_2CH_2SH$	+	−
CH_3CH_2SH	+	−
$CH_3CONHCHCH_2SH$ COOH	+	−
$H_2NCHCH_2SCH_3$ COOH	−	−

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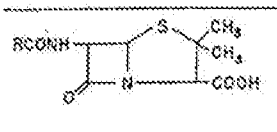
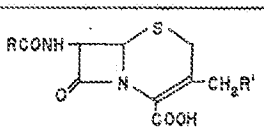
** Present address : Ball State University, Department of Chemistry, Muncie, Indiana, 47304, U.S.A.

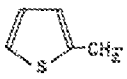
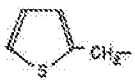
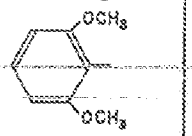
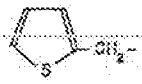
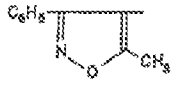
Table 2. Reaction of penicillins and cephalosporins with cysteine



Penicillin



Cephalosporin

R	Reactivity	R	R'	Reactivity
$C_6H_5CH_2$-	+	$C_6H_5CH_2$-	-H	-
$C_6H_5OCH_2$-	+	$C_6H_5OCH_2$-	-H	-
	+		-H	-
$C_6H_5CH(NH_2)$-	+	$C_6H_5CH(NH_2)$-	-H	-
	+		$-OCOCH_3$	-
	+	$C_6H_5CH(NH_2)$-	$-OCOCH_3$	-

hours, while under identical conditions the corresponding penicillins react completely within one hour.

In order to establish whether this difference in reactivity was related to the presence of a side chain on the antibiotics, 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) were treated with cysteine. After four hours the β-lactam band in the IR spectrum of the reaction mixture containing 6-APA and cysteine disappears. Under identical conditions there is no effect on the β-lactam of 7-ACA, since the control 7-ACA (lacking cysteine) shows an identical β-lactam (1800 cm^{-1}) to ester (1740 cm^{-1}) ratio as the reaction mixture.

The selectivity of this reaction indicates that penicillin β-lactam activity may be removed in the presence of cephalosporins. The results of the following experiment verified this contention. Cephalothin, 7-(2-thienylacetamido)cephalosporanic acid, was contaminated to the extent of 1% with phenoxymethylpenicillin. Samples were taken from a mixture of this cephalosporin, phenoxymethylpenicillin, and cysteine at pH 7.5 initially and after one and two hours. A microbiological assay of the one hour sample showed a trace amount of the original penicillin activity and after two hours the activity due to penicillin is gone. Mixtures of the same cephalosporin and phenoxymethylpenicillin without cysteine in pH 7.5 buffer and the same cephalosporin in buffer served as controls. Duplicates of all samples assayed were treated with penicillinase to illustrate the disappearance of penicillin activity by a known agent.

We have established that the reactivity of penicillenic acids, penicillins, and cephalosporins with 2-mercaptoamines differs. As a result, trace amounts of penicillin activity and, consequently, potential penicillin allergens can be destroyed in the presence of cephalosporins when more drastic physical treatment to remove possible contaminants would adversely effect cephalosporin activity. Studies are now in progress to determine whether structurally-modified cephalosporins interact with 2-mercaptoamines.

ysteine

	Reactivity
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	~
	~
	~
H_2	
H_3	~

lin, and cysteine at pH
one and two hours.
say of the one hour
race amount of the
tivity and after two
e to penicillin is gone.
ne cephalosporin and
lin without cysteine
nd the same cepha-
ed as controls. Dupli-
assayed were treated
lustrate the disappear-
vity by a known agent.
ed that the reactivity
penicillins, and cepha-
ercaptoamines differs.
amounts of penicillin
uently, potential peni-
be destroyed in the
rins when more drastic
remove possible con-
ersely effect cephalos-
es are now in progress
r structurally-modified
act with 2-mercapto-

Experimental

1. Phosphate Buffer Solution: KH_2PO_4 and Na_2HPO_4 (Mallinckrodt reagent) were used in the proper proportions for a 0.1 M pH 7.5 aqueous solution.

2. All measurements of the presence of benzylpenicillenic acid were made in aqueous buffer on a Beckman Model DB spectrophotometer using 1.00 cm quartz cells. Spectrophotometric determination of the disappearance of benzylpenicillenic acid was followed at 322 mμ.

3. All measurements of the presence of the β-lactam of penicillins and cephalosporins were made on mineral oil mulls of the lyophilized residue of the reaction mixtures on a Beckman Model IR 12 Double Beam Recording Infrared Spectrometer.

4. 2-Mercaptoamine Compounds: 2-Aminoethanethiol, cysteine, 2-mercaptoethanol, ethanethiol, N-acetyl-L-cysteine, and S-methylcysteine were available commercially.

5. Penicillins and Cephalosporins: 6-Aminopenicillanic acid, 7-aminocephalosporanic acid, benzylpenicillin, phenoxymethylpenicillin, 2-thienylmethylpenicillin, α-aminobenzylpenicillin, 2,6-dimethoxyphenylpenicillin, 4-(5-methyl-3-phenyl)isoxazolylpenicillin, 7-(2-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid, 7-(2-phenoxyacetamido)-3-methyl-3-cephem-4-carboxylic acid, 7-(2-thienylacetamido)-3-methyl-3-cephem-4-carboxylic acid, 7-(2-amino-2-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid, 7-(2-thienylacetamido)cephalosporanic acid, and 7-(2-amino-2-phenylacetamido)cephalosporanic acid were obtained commercially when possible or were synthesized in the Lilly Research Laboratories.

6. Reaction of Benzylpenicillenic Acid with 2-Mercaptoamines: The initial reaction mixture of 3.0 ml of 0.1 M phosphate buffer at pH 7.5 containing 1.0×10^{-3} M of the appropriate 2-mercaptoamine was placed in a stoppered quartz 1.00 cm Beckman cell. A solution of benzylpenicillenic acid in absolute ethanol (0.01 ml) was injected into the reaction cell, producing a concentration of 3.0×10^{-5} M. Optical density measurements were then recorded as a function of time.

7. Reaction of Penicillins and Cephalosporins with Cysteine: A reaction mixture of 50 ml of 0.1 M phosphate buffer at pH 7.5 containing 300 mg cysteine was added to a vessel containing 100 mg of the appropriate penicillin or cephalosporin. The resulting mixture was stirred at room temperature for four hours and subsequently frozen and lyophilized. Controls were run for each antibiotic tested which excluded the cysteine. The resulting residues were submitted for IR analysis of the presence of β-lactam at 1760~1810 cm^{-1}.

8. Reaction of 6-Aminopenicillanic Acid (6-APA) and 7-Aminocephalosporanic Acid (7-ACA) with Cysteine: Two reaction mixtures of 200 ml of 0.1 M phosphate buffer at pH 7.5 containing 3.0 g cysteine were added to two vessels containing 1.0 g 6-APA and 1.0 g 7-ACA, respectively. A third reaction mixture of 200 ml of 0.1 M phosphate buffer at pH 7.5 containing 1.0 g 7-ACA served as a control. All three reaction mixtures were stirred at room temperature for four hours and subsequently frozen and lyophilized. The resulting residues were submitted for IR analysis of the presence of β-lactam at 1760~1810 cm^{-1} and ester at 1740 cm^{-1} in the case of 7-ACA.

9. Reaction of Phenoxymethylpenicillin with Cysteine in the Presence of a Cephalosporin. Isolation and Analysis of Penicillin: Three samples (labelled A, B, and C) were prepared; each contained 100 ml of 0.1 M phosphate buffer. Sample A contained 1.0 g of 7-(2-thienylacetamido)cephalosporanic acid, 10 mg of phenoxymethylpenicillin, and 400 mg cysteine; sample B contained 1.0 g of the same cephalosporin and 10 mg of phenoxymethylpenicillin; and sample C contained the cephalosporin in buffer and served as a control. Samples were taken from samples A, B, and C initially and after one and two hours and added to pH 3.0 water washed diisopropyl ether (DIPE). The pH of the aqueous phase was adjusted to 1.5 with 20% H_2SO_4 solution. The resulting slurry was filtered on 7 cm Whatman No. 1 filter paper on a Buchner funnel. The filtrate was placed in a separatory funnel

and 100 ml of the DIPE layer was applied to 1/4" × 20" sheets of Whatman No. 4 paper which had been previously buffered with 0.5 M to 0.75 M pH 6.2 phosphate buffer. The chromatogram was allowed to equilibrate in a solvent chamber for approximately two hours before adding water saturated diethyl ether to the solvent tray. The chromatogram was allowed to develop down flow for approximately three hours followed by development on *Sarcina lutea* (Agar pH was 6.7 prior to pouring and plates were allowed to stand overnight in refrigerator prior to use). The appropriate standards were used for comparison of active material and duplicate samples were treated with penicillinase to illustrate the disappearance of penicillin activity by a known agent. The limit of detection was 0.5 ppm.

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